

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Appellants: Marc PREAUDAT et al.

Examiner: SHEN, Bin

Serial No.: 10/522,909

Group Art Unit: 1657

Filed: October 5, 2005

Confirmation No.: 7128

Title: METHOD FOR DETERMINING ENDOGLYCOSIDASE ENZYME ACTIVITY

BRIEF ON APPEAL UNDER 37 C.F.R. § 41.37

Mail Stop Appeal Brief- Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is an appeal from the decision of the Examiner finally rejecting claims 36, 38-40, 42-48 and 127-134 of the above-identified application.

(I) REAL PARTY IN INTEREST

CIS Bio International, Saclay, FRANCE is the Assignee of Record of the entire right, title, and interest in and to the above-identified application, as recorded in the U.S. Patent and Trademark Office on June 19, 2006, at Reel/Frame 018215/0975.

(II) RELATED APPEALS AND INTERFERENCES

Appellants, their legal representative and the assignee are not aware of any related appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the instant appeal.

(III) STATUS OF THE CLAIMS

Claims rejected: Claims 1, 2, and 5-12.

Claims allowed: (none)

Claims canceled: (none)

Claims withdrawn: Claims 3, 4, and 13-18

Claims on Appeal: Claims 1, 2, and 5-12 (Copy of claims on appeal in attached Appendix).

(IV) STATUS OF AMENDMENTS

A Reply after Final Rejection containing claim amendments was filed on June 21, 2007,

after the Final Rejection. The Advisory Action mailed July 12, 2007, indicates that the amendments therein will be entered for purposes of this appeal. Thus, the amendments are entered and are reflected in the claims on appeal shown in the attached Appendix of Claims.

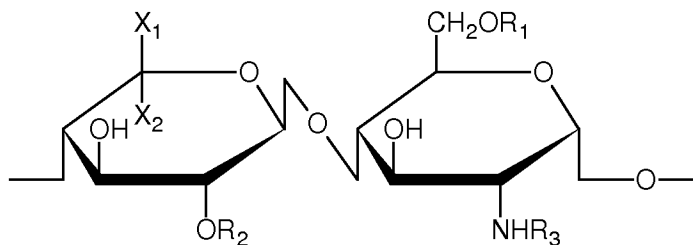
(V) SUMMARY OF CLAIMED SUBJECT MATTER

Appellants' invention (sole independent claim 1) is directed to a method for determining endoglycosidase enzyme activity in a sample; see, e.g., page 1, lines 4-5, page 4, lines 11-15, and page 5, lines 14-16, of the instant specification. The method comprises

- i. bringing a substrate which can be cleaved by said endoglycosidase into contact with said sample, and
- ii. measuring a change in the amount of intact substrate,
a decrease in the amount of this substrate being representative of endoglycosidase activity in the sample,

wherein the substrate is directly or indirectly labeled with a first donor compound and with a second acceptor compound, and the amount of intact substrate is determined by measuring a signal emitted by the acceptor compound, this signal resulting from a transfer, via a close proximity effect, between the donor and the acceptor; see, e.g., page 7, lines 6-15, of the instant specification.

Appellants' claim 6 and the claims dependent therefrom are drawn to substrate molecules that are examples of with the aforementioned assay method. The substrate is a heparan sulfate proteoglycan or a derivative thereof, extracellular matrix-associated heparan sulfate or a derivative thereof, heparin, or heparan sulfate or a derivative thereof, containing from 1 to 30 units of formula:



wherein

R₁ and R₃ are, independently of one another, H, SO₃H, or SO₃H⁻,

R₂ is SO₃H, SO₃H⁻, or C(O)CH₃, and

X₁ and X₂ are, independently of one another, H or COOH.

See, for example, page 9, lines 4-16 of the originally-filed specification.

(VI) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following sole remaining ground of rejection is requested to be reviewed on appeal.

For each ground, any separate grouping of the claims subject to that rejection is indicated.

The rejection of claims 1, 2, and 5-12 under 35 U.S.C. §103, as being obvious over Bazin (*Spectrochimica Acta*, vol. 57, pages: 2197–2211, 2001) in view of Nicholson (US 4,859,581).

Claims 1, 2, and 5-12, on appeal, are grouped together.

(VII) ARGUMENT

Claims 1, 2, and 5-12, on appeal, are not obvious to one of ordinary skill in the art from Bazin (*Spectrochimica Acta*, vol. 57, pages: 2197–2211, 2001) in view of Nicholson (US 4,859,581). The rejection thereof under 35 U.S.C. §103 is not supported on the record as a whole and should be reversed.

The claimed invention is directed to a method for assaying enzymatic activity using specific substrates and analyzing the products that are generated in the process. As will be detailed below, the prior art can be summarized as follows and its consideration as a whole fails to render the claimed invention obvious to one of ordinary skill in the art:

As outlined in §1.2 of the publication, Bazin is drawn to the use of Europium cryptate and allphycocyanin as donor and acceptor compounds in homogenous time-resolved fluorescence (HTRF) assays of peptide substrates. In §3.1 at page 2205, Bazin expressly states that the substrates used in these assays are proteins, and the assay is primarily used in the measurement of protein-protein interactions. This aspect is further described in §3.2 and §3.3 of the reference. In this context, §3.2.2 describes the use of this method in a protease assay wherein “a peptide containing a sequence recognized by a protease is labeled on one side with XL665 (allphycocyanin) and on the other side with biotin [and] in presence of the protease, which cleaves the peptide, the signal is measured.” Bazin does not teach or suggest the substrate molecules described in the instant application, nor an assay technique which is catered to such substrate molecules and/or products thereof.

Nicholson (US 4,859,581) is drawn to a chromatographic method of detection of fluorescein or chromophore-labeled heparanase sulfate (HS). See, the paragraph bridging cols. 4 and 5 of Nicholson and the disclosure contained in the Figures. In the paragraphs bridging col. 8, line 30 to col. 9 line 2, Nicholson exemplifies such tagged HS substrates. It is explicitly taught that HS is “tagged at one end, preferably the reducing end, to another molecule.” Such tags have the “ability to act as a potential ‘handle’ for the labeled glycosaminoglycan chain and for the residue of the glycosaminoglycan chain remaining after cleavage by a glycosaminoglycan endoglycosidase.” The ‘handle’ would be “able to act as a point of attachment for a protein molecule having affinity for the bound tagging molecule.” There is no mention of molecules that are labeled with two compounds. Furthermore, Nicholson is absolutely silent with respect to donor-acceptor pairs which are in close proximity to one another.

As for the method of detection, Nicholson’s assay system is devoted to the use of

chromatographic techniques. It is stated that such labeled molecules allow “rapid analysis of degradation fragments on HPLC equipped with a flow fluorescence detector.” The cited reference does not describe a method for assaying for endoglycosidase activity by utilizing a substrate which is directly or indirectly labeled with a first donor compound and with a second acceptor compound, let alone wherein the amount of intact substrate is determined by measuring a signal emitted by the acceptor compound, this signal resulting from a transfer, via a close proximity effect, between the donor and the acceptor compounds. Moreover, Nicholson requires “separation of the reaction products from the substrates based on their size.” In contrast, Appellants’ claimed invention is not based on size-resolution.

According to the MPEP, to establish a *prima facie* case of obviousness, the prior art reference (or references when combined) must teach or suggest all the claim limitations. See, M.P.E.P. §2143. Bazin fails to provide any hint or suggestion to one of ordinary skill that reagents and techniques devoted for the assessment of *protease* activity could be utilized for the assessment of the activity of another distinct class of enzymes (i.e., endoglycosidase). Nicholson is silent with respect to substrate molecules having labels which comprise a donor and acceptor pair and utilizing FRET-based assays for studying enzymes that cleave such molecules. As such, a combination of Bazin and/or Nicholson, even at their broadest interpretation, fails to teach all the elements of Appellants’ claims. There is no mention of employing a FRET-based assay technique using sugar substrates for the measurement of endoglycosidase activity. Therefore, a combination of Nicholson and Bazin cannot render obvious the subject matter of the instant invention.

The claimed invention is further unobvious in view of a skilled worker’s knowledge of substrate molecules taught by the instant invention and assay techniques devoted to the utilization of such substrates. In this regard, Bazin utilizes substrate molecules whose chemical structures are known and which are specifically engineered so the donor and acceptor fluorophores will both bind at known positions on the substrate. The peptide substrates in Bazin are engineered to be labeled “on both ends” and “at known positions.” See, the disclosure contained in Bazin’s exemplified substrate molecules, wherein, peptide substrates are engineered to achieve close proximity of fluorescent labels, which when cleaved, allows for a change in FRET signal to be detected. The following paragraphs of the cited reference provide such a disclosure:

p. 2205, 1st column, last paragraph: “Cummings and coworkers built up an HIV protease format by tagging both ends of a peptide respectively with biotin and a sequence containing phosphotyrosine used here as a tag.”

p. 2205, 2nd column, 1st paragraph: "...Takemoto et al performed a Caspase 3 indirect assay. A7 amino-acid peptide was labeled at both ends with biotin and DNP groups"

p. 2206, 3.2.2 "A peptide containing a sequence recognized by a protease was labeled on one side with XL665 and on the other side with biotin. (Emphasis added)

Therefore, Bazin et al., as far as proteases are concerned, teach that the enzyme substrate must be labeled – either directly or indirectly – "on both ends" (i.e., both sides) of the cleavage site. The present invention is not limited by such engineering techniques.

A skilled artisan understands that a peptide is made of a known combination of the 20 natural amino-acids; tools for peptide analysis are generally well-described in the art, as well as techniques relating to chemical synthesis of such peptide molecules. Moreover, proteases cleave peptide substrates at known locations and sequences. When developing a FRET-based protease assay, the skilled artisan understands that there is little left to chance: the cleavage site of the peptide and the coupling-site of the donor and acceptor fluorophore are to be precisely known. However, the substrates of the instant invention, for example, heparan sulfate proteoglycan, ECM-associated heparan sulfate, heparin, or heparan sulfate, containing from 1 to 30 units of glycosidic monomers, are structurally distinct from the substrates claimed by Bazin et al. Absent knowledge or guidance that FRET-based assays can be utilized for the assessment of structurally distinct class of endoglycosidase enzymes which act on glycosidic substrates, there would be no motivation to combine the references. In view of Bazin et al. one would not have thought this was possible. For a FRET-based glycosidase assay, the substrate is not a well-characterized peptide, but a mix of heparan sulfates (HS) or HS proteoglycans (HSPG) of different and unknown sequences and structures. This results in the fact that neither the cleavage site nor the exact position of the donor and acceptor fluorophore can be wholly presumed in such substrate molecules.

Heparan sulfate (HS) is a linear polysaccharide consisting of a chain of disaccharide units of N-acetyl-D glucosamine that is linked to D-glucuronic acid. The disaccharide repeat units *can be* (but are not necessarily) modified to include N- and O- sulfation (6-O and 3-O sulfation of the glucosamine, and 2-O sulfation of the uronic acid) and epimerization of beta-D-glucuronic acid to alpha-L-iduronic acid. Together, the five different modifications for disaccharides give rise to 32 combinations. Thus, the complexity of heparan sulfate is even greater than that of proteins, which are made up of 20 aminoacids. With these 32 building blocks, a heparan sulfate octasaccharide could have over a million possible sequences.

According to Codée (Drug Discovery Today: Technologies, vol. 1, pp. 317-326, 2004), "the highly complex structure of heparan sulfate presents a formidable synthetic challenge and the incorporation of the full array of variations in oligosaccharides of significant length is a

daunting task." See, the INTRODUCTION section of Codée et al (2004). Moreover, Codée teaches that "it is virtually impossible to obtain specific heparan sulfate from natural sources owing to their microheterogeneity." See, for example, the disclosure contained in col. 2, at page 317 of Codée et al.

Consequently, the teaching of Bazin et al., which is drawn to FRET-based assay of protease activity, is not applicable to a FRET-based assay of an endoglycosidase activity. Since the skilled artisan appreciates the nature of the substrate compounds involved in the assay of such endoglycosidases, for example, heparan sulfate substrate and the like, and further has no knowledge on labeling of this substrate "on both ends" (i.e., on both sides of a cleavage site), the simplicity of Bazin's methods and substrates cannot be applied towards the claimed technique of endoglycosidase assay.

Furthermore, Appellants submit that a skilled artisan would also appreciate differences between heparanase assay as compared to the heparitinase assay, as recited in the instant claims. See, for example, Appellants' claim 5. For example, additional technical challenges appear with heparanase enzymes: Heparanase cleaves HS into large fragments, as compared with other HS hydrolase such as heparitinase. Consequently, there are less cleavage sites for this enzyme, which increases the technical difficulty hurdle of labeling the substrate "on both sides" of a cleavage site. The added functional complexity of the claimed enzymes, and the technical complexity of assaying for such, is not possible with the generic chromatographic technique disclosed by Nicholson. Evidence of such unexpected properties further supports non-obviousness. The advantages, for example, assay sensitivity and ease of use, are surprising and convincing evidence for patentability over what is known in the art. Under all of these circumstances, a rejection under 35 U.S.C. §103 is not supportable.

For all of the above reasons, it is urged that the decision of the Examiner rejecting claims 1, 2, and 5-12, on appeal, is in error and should be reversed.

The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,

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Attorney Docket No.: **LOM-0047**

Date: September 24, 2007

(VIII) CLAIMS APPENDIX

Claim 1. A method for determining endoglycosidase enzyme activity in a sample, comprising:

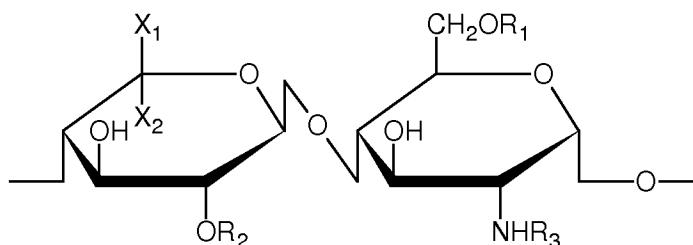
- i. bringing a substrate which can be cleaved by said endoglycosidase into contact with said sample, and
- ii. measuring a change in the amount of intact substrate, a decrease in the amount of this substrate being representative of endoglycosidase activity in the sample,

wherein the substrate is directly or indirectly labeled with a first donor compound and with a second acceptor compound, and the amount of intact substrate is determined by measuring a signal emitted by the acceptor compound, this signal resulting from a transfer, via a close proximity effect, between the donor and the acceptor.

Claim 2. The method as claimed in claim 1, wherein the first donor compound and the second acceptor compound are fluorescent compounds, the close proximity transfer is an energy transfer, and the signal emitted is a fluorescent signal.

Claim 5. The method as claimed in claim 1, wherein the endoglycosidase is an enzyme of the heparanase type which is recombinant heparanase, purified heparanase, nonpurified heparanase or heparitinase.

Claim 6. The method as claimed in claim 1, wherein the substrate is a heparan sulfate proteoglycan or a derivative thereof, extracellular matrix-associated heparan sulfate or a derivative thereof, heparin, or heparan sulfate or a derivative thereof, containing from 1 to 30 units of formula:



wherein

- R₁ and R₃ are, independently of one another, H, SO₃H, or SO₃H⁻,
R₂ is SO₃H, SO₃H⁻, or C(O)CH₃, and
X₁ and X₂ are, independently of one another, H or COOH.

Claim 7. The method as claimed in claim 6, wherein the substrate is covalently attached to a donor fluorescent compound and to an acceptor fluorescent compound.

Claim 8. The method as claimed in claim 6, wherein the substrate is covalently attached to a member of a first ligand-receptor pair and to a member of a second ligand-receptor pair, and wherein the donor fluorescent compound is covalently attached to the other member of the first ligand-receptor pair and the donor fluorescent compound is attached to the other member of the second ligand-receptor pair.

Claim 9. The method as claimed in claim 6, wherein the substrate is covalently attached to the donor fluorescent compound and is covalently attached to a member of a ligand-receptor pair, and the acceptor fluorescent compound is covalently attached to the other member of said ligand-receptor pair.

Claim 10. The method as claimed in claim 6, wherein the substrate is covalently attached to the acceptor fluorescent compound and is covalently attached to a member of a ligand-receptor pair, and the donor fluorescent compound is covalently attached to the other member of said ligand-receptor pair.

Claim 11. The method as claimed in claim 8, wherein the first and the second ligand-receptor pair are different and are chosen from the pairs: hapten/antibody, DNP/anti-DNP antibody, GST/anti-GST antibody, biotin/avidin, 6HIS/anti-6HIS antibody, Cmyc/anti-Cmyc antibody, FLAG/anti-FLAG antibody, or HA/anti-HA antibody.

Claim 12. The method as claimed in claim 1, wherein the donor compound is a rare earth cryptate or chelate, and the acceptor fluorescent compound is a rhodamine, cyanin, squaraine, bodipy dye, fluorescein, allophycocyanin or a derivative thereof.

(IX) Evidence appendix

Appendix of evidence submitted pursuant to §§ 1.130, 1.131, or 1.132 of this title or of any other evidence entered by the Examiner and relied upon by appellant in the appeal, along with a statement setting forth where in the record that evidence was entered in the record by the Examiner. Copies of the evidentiary document are attached.

Reference	Entered in the Record
1. Codée et al., <i>Drug Discovery Today: Technologies</i> , vol. 1, pp. 317-326, 2004 [ABSTRACT].	Filed by appellants with Reply of June 21, 2007; No note of consideration made in the Advisory Action mailed July 12, 2007.

(X) RELATED PROCEEDINGS APPENDIX

(None)

(XII) Appendix of Comments to Pre-Appeal Conferees

(None)



DRUG DISCOVERY
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Lead optimization

The synthesis of well-defined heparin and heparan sulfate fragments

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Heparin and heparan sulfate are key players in a plethora of physiological processes. Organic synthesis is the method of choice for the production of these oligosaccharides and their derivatives and analogues. The highly complex structure of these polysaccharides presents a formidable synthetic challenge and the incorporation of the full array of variations in oligosaccharides of significant length is a daunting task. This review records the development of strategies to access these exciting biomolecules.

Introduction

Heparin and heparan sulfate (H/HS, Fig. 1a) are negatively charged, unbranched carbohydrate polymers and present the most complex members of the glycosaminoglycan (GAG) superfamily, which further includes dermatan sulfate, chondroitin sulphate, keratan sulfate and hyaluronic acid. H/HS are built up from alternating glucosamine and uronic acid (D-glucuronic or L-iduronic) residues, which can be highly functionalised: O-sulfation can occur on the 2-position of the uronic acids and the 3- and 6-position of the glucosamine while the amino functions might be sulfated, acylated or unsubstituted. H/HS-polysaccharides interact with over a hundred proteins and have a crucial position in the regulation of various physiological processes [1]. To understand the mode of

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Difficulties in the isolation of well-defined heparin and heparan sulfate fragments have led to the use of organic synthesis for the production of such oligosaccharides. Here, Codée et al. review several synthetic methodologies and target-oriented and modular approaches that are used for the development of heparin and heparan sulfate fragments. van Boeckel was involved in a long-standing joint venture between Organon and Sanofi, generating novel synthetic heparin antithrombotics. As an adjunct professor he collaborated for many years with the other authors, who are experts in carbohydrate chemistry at Leiden University. The group is currently headed by Professor Herman Overkleef. Jeroen Codée has recently completed his PhD; his thesis describes new synthetic methodologies of glycosaminoglycans in solution and on solid support.

action of H/HS at a molecular level it is of prime importance to have access to pure, well-defined H/HS-fragments. Because it is virtually impossible to obtain specific H/HS-structures from natural sources (owing to their microheterogeneity), organic synthesis has become the method of choice for the production of GAG-oligosaccharides as well as their derivatives and analogues [2–5]. This review focuses on the synthesis of H and HS, because (1) they are the most complex members of the GAG superfamily and (2) the most important technological and methodological advancements in the field of GAG-synthesis have come from the research on H/HS-assembly.

Synthetic methodologies

The synthesis of H/HS-polysaccharides presents several challenges, imposed by the complex structure of the target mole-

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Glossary

Modular approach: a synthetic strategy that employs closely related building blocks (modules). The similarity of the building blocks should allow for standardized condensation reactions and provide flexibility and variety in use.

Non-glycosamino glycans: synthetic glycosaminoglycans which bear O-methyl functions instead of free hydroxyls and O-sulfate groups in place of N-sulfates

cules [2–5]. First, L-idose and L-iduronic acid are not readily available from commercial or natural sources, and therefore efficient synthetic routes to access sufficient amounts of these monosaccharides are required. The next challenge is the development of a suitable protecting-group strategy to allow the implementation of the high degree of functionalization of the H/HS fragments (Fig. 1b). In the vast majority of H/HS syntheses, the following sequence of events completes the construction of the H/HS-fragment: (1) selective deprotection of the hydroxyls to be sulfated; (2) sulfation of these hydroxyls; (3) deprotection of the remaining alcohol and amino functions; and (4) selective sulfation of the free amines. Generally to accommodate these manipulations in the synthesis, the free and sulfated alcohol functions are masked as benzyl ethers and ester groups (e.g. acetyl, benzoyl and pivaloyl), respectively. The amino functions are often taken through the synthesis as the corresponding azides. Next to these protecting groups an additional set of orthogonal groups (e.g. silyl ethers, levulinoyl esters, 9-fluorenyl methoxy carbonyl carbonates, *p*-methoxybenzyl and allyl ethers) is required to temporarily block the reducing and/or non-reducing termini. The last challenge, imposed by the H/HS structure, is the stereoselective and efficient formation of the interglycosidic bonds in the carbohydrate backbone. For the stereoselective formation of the α -glucosamine linkages, a non-participating group at C-2 (e.g. azide) of the glucosamine building block is required, whereas the C-2 of the uronic acid motives should be equipped with a participating acyl function. The presence of the carboxylate functions is of profound influence on the efficiency of the glycosylation reactions, because the electron-withdrawing carboxyl group at C-5 not only significantly reduces the nucleophilicity of the proximal OH-4 group but also lowers the glycosyl-donor reactivity of the uronic acid building blocks compared with their non-oxidised counterparts.

Target-oriented approaches

The first efforts towards the assembly of H/HS fragments were directed towards the synthesis of the anticoagulant heparin pentasaccharide (**1**), also known as the fragment DEFGH (Fig. 1b, structure **1**) [4,5]. This unique pentasaccharide sequence binds antithrombin III (AT III), a crucial inhibitor for the blood coagulation factors IIa (thrombin)

and Xa (Fig. 1c,d). Whereas the pentasaccharide fragment is sufficient for inhibition of factor Xa, the inhibition of thrombin requires a longer heparin chain (>16 monosaccharides). In the latter case, the heparin chain functions to bind both AT III and thrombin in a ternary complex thereby guiding the protease (thrombin) towards the reactive center loop of its inhibitor (AT III) (Fig. 1d). Epic total syntheses of the highly functionalized oligosaccharide **1** were reported in the mid-1980s by the groups of Sinaý [6] and van Boeckel [7]. Ever since, a vast array of analogues has been synthesised to establish the SAR of the pentasaccharide domain [4,5]. One of the first analogues to be synthesised was pentamer **2** (Fig. 1b), the α -O-methyl glucoside analogue of **1** [8], which was recently launched as a novel antithrombotic drug by the pharmaceutical companies of Organon (<http://www.organon.com/>) and Sanofi (<http://www.sanofi-aventis.com/>) under the name Arixtra® (Fondaparinux). Capping the anomeric center of unit H with a methyl group simplifies the synthesis and purification of the saccharide significantly because the reactive aldehyde group at the reducing end is persistently protected. The initial syntheses of pentasaccharides **1** and **2** were based on the state-of-the-art oligosaccharide synthesis at the time and used glycosyl bromide and orthoester building blocks (**6–10**) in combination with the protecting-group strategy outlined above and required over 60 steps (retrosynthetically depicted in Fig. 1b). In the course of the development of Arixtra®, the synthesis of **2** has improved using building blocks **11** and **12**, as depicted in Fig. 1b, and much chemistry has been adjusted to facilitate a factory process. Following a target-oriented strategy designed for these pentasaccharides, a collection of analogues has been constructed including decarboxylated [4], phosphorylated [9], epimeric [4], flexible [4], desulfated [10] and supersulfated [4] analogues. The biological activities of these analogues point to the structure activity relationships depicted in Fig. 1b. Furthermore, using tailor-made conformationally restricted pentasaccharides, it was established that the L-iduronic acid moiety (a rather flexible motive in the DEFGH-sequence) has to adopt the 2S_0 -conformation to exert its biological activity [11].

In the pentasaccharide field, an important synthetic step forward has been the development of the “NON-GLYCOSAMINO” GLYCAN (see Glossary) heparin oligosaccharides (e.g. **3** and **4**, Fig. 1b), which bear O-methyl functions instead of free hydroxyls and O-sulfate groups in place of N-sulfates (technology developed by Organon N.V. and Sanofi-Aventis) [12]. These modifications dramatically simplify the assembly of H/HS-like fragments because (1) the deprotection and sulfation sequence at the end of the oligosaccharide is more straightforward and requires less steps; (2) glucose instead of glucosamine building blocks can be used; and (3) a larger choice of protecting groups is available. In addition, the last two issues

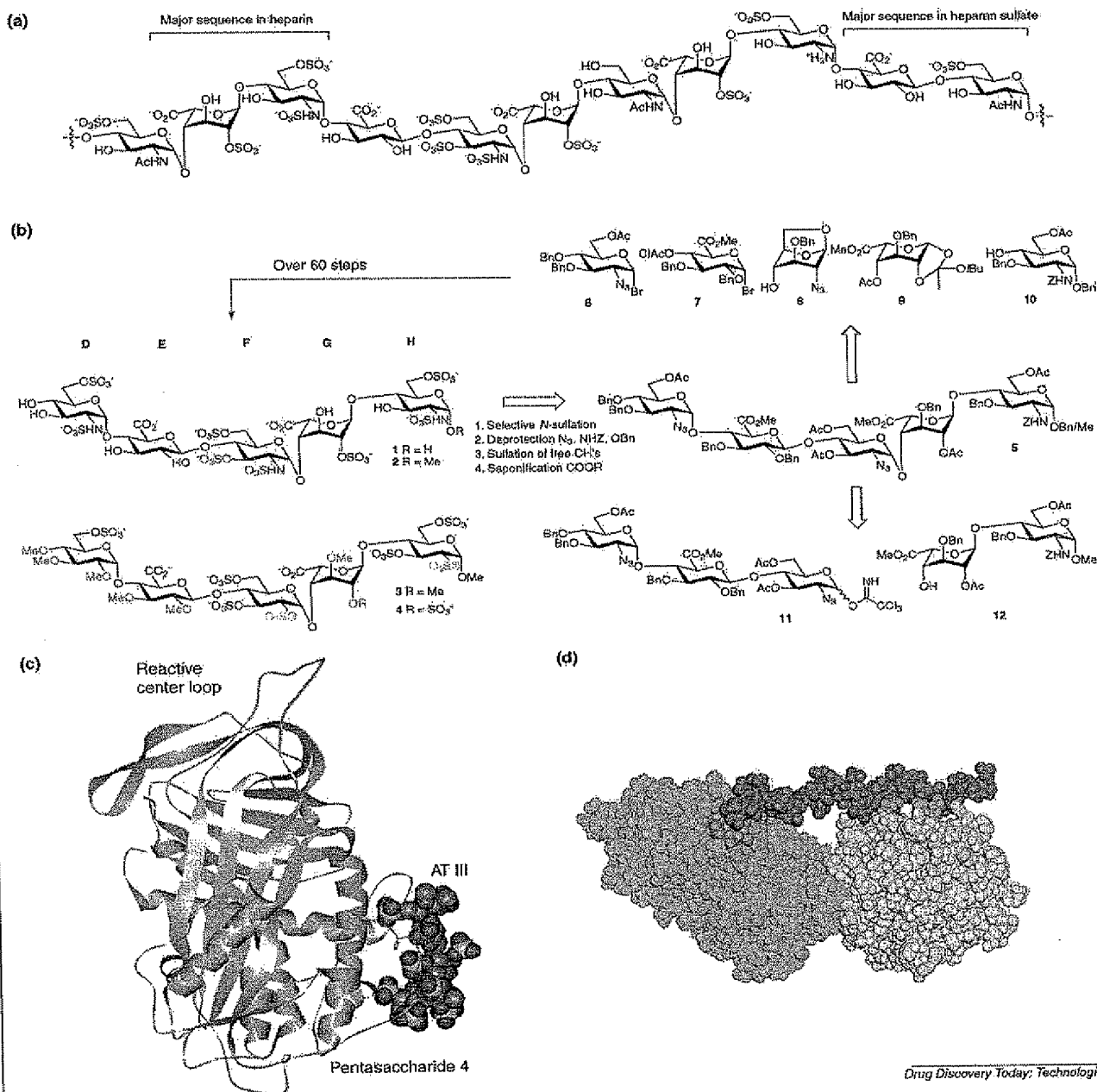


Figure 1. The anticoagulant heparin pentasaccharide story. (a) Heparin/heparan sulfate (H/HS) polysaccharide. H and HS differ in substitution pattern (H contains on average 2.7 and HS only 1 sulfate group) and glucuronic to iduronic acid ratio (for H: 1:9, for HS: 6:4). (b) Synthetic strategy used for the synthesis of the H-pentasaccharides 1 and 2. Groups in 1 and 2 highlighted in red are essential for antithrombin III (AT-III) activation, whereas groups depicted in blue contribute to biological activity. The non-GAG analogues (3 and 4) bear *O*-methyl groups (in green) and *O*-sulfate groups (orange) instead of free hydroxyls and *N*-sulfates, respectively. (c) Crystal structure of AT-III in complex with non-GAG pentasaccharide 4 [33]. (d) Molecular model of the ternary AT-III–heparin–thrombin complex, which shows that heparin forms a bridge between the two proteins. Six to eight monosaccharide residues do not interact with the proteins [34].

have a major impact on the reactivity of the donor and acceptor building blocks in the glycosylation reactions *en route* the target oligosaccharide. Besides these synthetic advantages (the total synthesis of **3** comprises approximately 25 steps), the non-GAGs have also provided more potent pentasaccharide analogues. Pentamer **3** (Idraparinux) has an anti-Xa activity of 1600 U/mg as opposed to the reactivity of 700 U/mg of the “natural” pentamer **2**. Idraparinux is in late clinical trials and because of its high affinity to AT III has a very long half-life in circulation which allows once a week administration only [13].

The more straightforward access to non-GAGs compared with GAGs is nicely illustrated by the synthesis of the impressive polysaccharides **15** [14] and **20** [15] (Fig. 2a). Eicosamer **15** was synthesized to study the interaction with both AT III and thrombin (factor IIa) via a ternary complex. Therefore, the polysaccharide had to contain both an AT III binding domain (ABD) and a thrombin-binding domain (TBD) separated by a spacer segment. At the time of synthesis it was unknown which terminus (the reducing or non-reducing) of the ABD had to be extended with the spacer moiety and the TBD. Therefore, oligosaccharides were synthesized which can be regarded as a continuum of ABDs. A hexasaccharide (**15**), which consisted of three identical iduronic acid–glucose disaccharide motives was selected as an ABD domain. This made it possible to synthesize the oligomers using one single dimer building block (i.e. **13**) in an iterative fashion. Employing this building block a hexa-, deca-, dodeca-, tetradeca-, hexadeca-, octadeca- and eicosasaccharide were assembled, of which the latter (**15**) proved to be the most potent thrombin inhibitor. Dreef-Tromp *et al.* [16] used similar imidate building blocks (e.g. **17**) in the first polymer supported synthesis of non-GAG heparin oligomers (Fig. 2b). The soluble monomethyl polyethylene glycol support (MPEG) was employed to synthesize a set of heparin oligosaccharides (**19**), which differed in length (up to 12-monomers) and sulfation pattern.

Another milestone was achieved by the impressive synthesis of the thrombin inhibiting heptadecasaccharide (**20**) (Fig. 2c) [15]. This non-GAG oligosaccharide is composed of an ABD that is connected at its non-reducing end to a TBD with a neutral oligosaccharide spacer. This extraordinary molecule was shown to be more potent than standard heparin and low-molecular mass heparin in models for both venous and arterial thrombosis. Furthermore, it showed a strong decrease of non-specific interactions with basic proteins (and hence less side effects, e.g. heparin-induced thrombocytopenia) as a result of the incorporation of the neutral spacer motive.

Modular approaches

MODULAR APPROACHES (see Glossary) developed for the assembly of non-GAG heparin polysaccharides such as **15** and **19** have

also been applied for the synthesis of the synthetically more challenging glycosaminoglycan H/HS. A modular strategy that is applicable to a variety of (dimer) building blocks, differing in their substituent pattern (acyl versus benzyl protecting groups, differentiated amino functionalities, iduronic versus glucuronic acid configuration) would open up the way to tackle the enormous diversity found in H/HS structure. Furthermore (automated) solid-phase protocols can then be envisaged to speed up the process of H/HS-oligosaccharide assembly. A variety of different approaches has been reported over the years, differing in the use of protecting groups in the building blocks, timing of the oxidation step to access the uronic acids, and glycosylation procedure [17–23]. However, no general strategy has evolved thus far that accommodates all of the characteristics displayed by the H/HS-oligosaccharides. The incorporation of glucuronic acid residues and the differentiation of the OH-2 of the uronic acids and the amino functionalities have only very scarcely been reported.

One of the first modular approaches, as reported by Tabeur *et al.* [17], was directed towards the assembly of oligomers corresponding to the regular sequence of heparin. The synthetic strategy, based on the use of dimer **21** (Fig. 2d), which bears close resemblance to the non-GAG building blocks **13** (Fig. 2a) hinges on the reliable stereoselective formation of the α -D-glucosazide-1-iduronic acid linkage.

The Martín-Lomas laboratory has focused on the elucidation of the activation mechanism of the fibroblast growth factors (FGFs) by glycosaminoglycans (Fig. 3a). Regular-sequence heparin oligosaccharides (e.g. hexamer **30**) were prepared following the strategy depicted in Fig. 3b [18]. In this approach, the pivotal building block consisted of a disaccharide having the glucosazide motive at its non-reducing end (**24** and **28**). The stereoselective formation of the interglycosidic iduronic acid–glucosamine bonds was guided by the 2-O-pivaloyl and 2-O-benzoyl groups, providing anchimeric assistance in the condensations. The OH-4' function was liberated after each coupling event by cleavage of the benzylidene group, and ensuing regioselective benzylation. Interestingly they also constructed fragments, having differentiated amino substituents, bearing either acetyl or sulfate groups (e.g. structure **35**, Fig. 3c) [22]. Incorporation of the *N*-acetyl glucosamine moieties was accomplished using building block **36**, a close analogue of dimer **24**. This building block proved to be a poor acceptor glycoside, thereby illustrating one of the remaining major challenges in heparin synthesis: the efficient incorporation of differently functionalised amino groups.

The Martín-Lomas laboratory extended their solution-phase chemistry to a solid-phase approach (see Fig. 3b) [25]. At first glance this might seem a trivial operation, but the use of building blocks of low reactivity seriously limits the possibilities for such a translation. Hence, a soluble solid

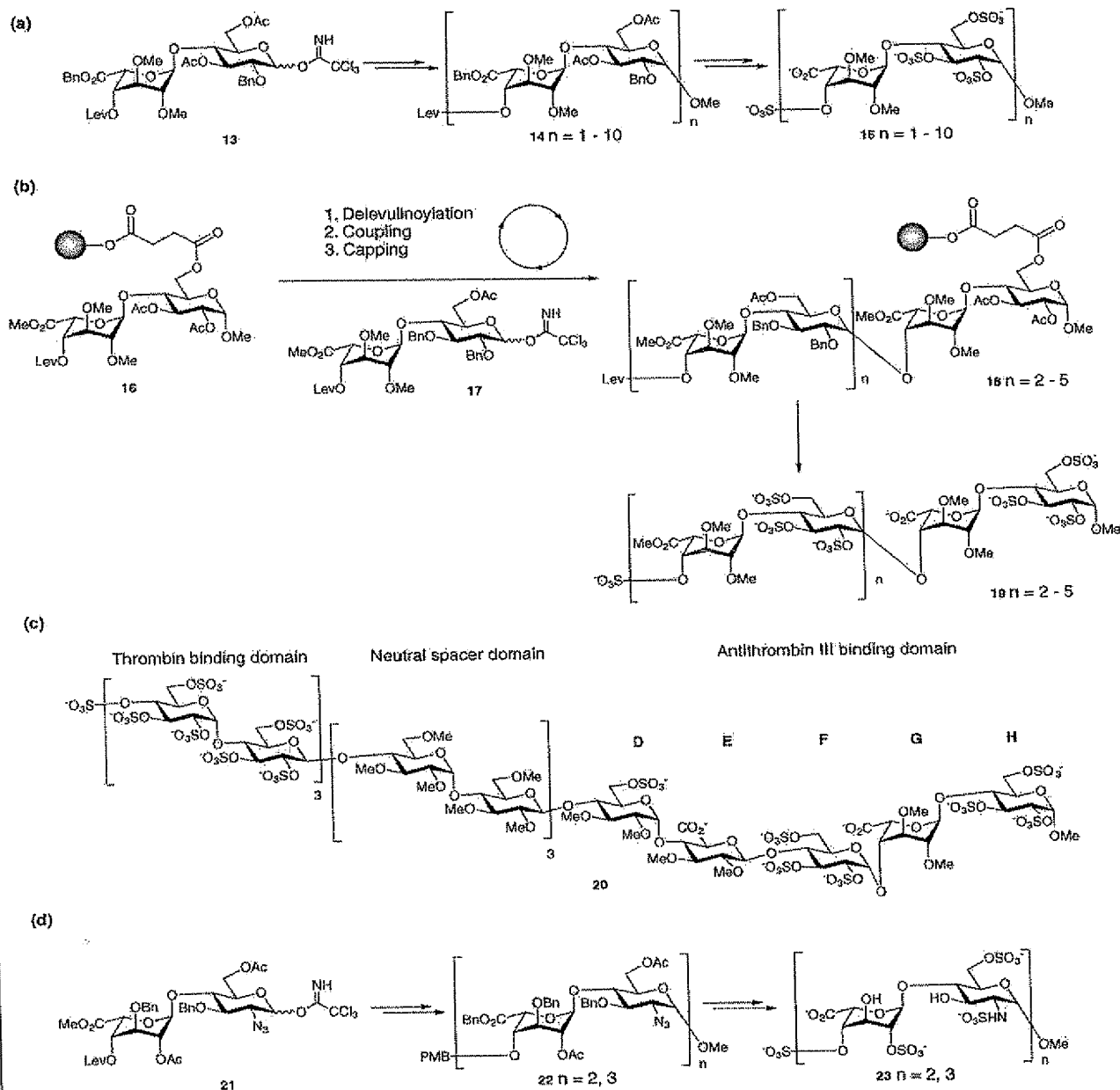
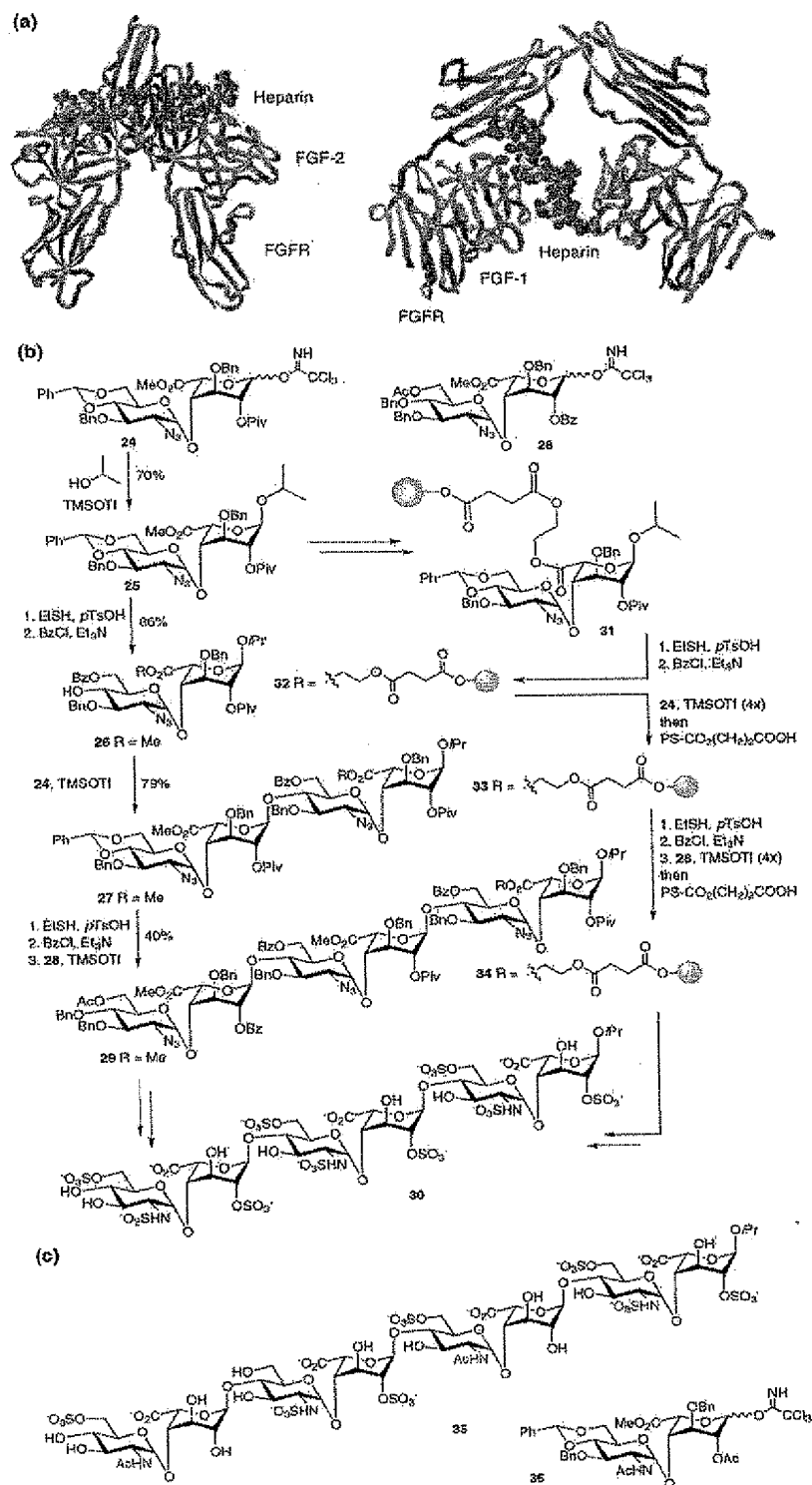


Figure 2. Synthetic approaches towards heparin-like oligosaccharides. (a) Modular approach towards an antithrombin active non-GAG heparin oligosaccharide. (b) First polymer support synthesis of non-GAG heparin oligosaccharides. (c) Synthetic thrombin inhibiting heparin-like heptadecasaccharide without side effects. (d) Modular approach towards the assembly of glycosaminoglycan heparin oligosaccharides.

support (polyethylene glycol ω -monomethyl ether, MPEG) was chosen. A succinyl linker system, analogous to the linker used in the MPEG-based non-GAG synthesis of Dreef-Tromp *et al.* [16], was used for the attachment of the first disaccharide to the polymer. The crucial glycosylation events were ex-

cuted using two equivalents of the imidate donor **24** or **28** in four repetitive cycles. Subsequently, the unreacted acceptor hydroxyls were scavenged with an acid functionalized solid support. This rather elaborate coupling cycle clearly indicates the low reactivity of the system. The partially protected



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Table 1. Comparison summary table

	Technology 1	Technology 2	Technology 3
Name of specific type of technology	Solution-phase synthesis GAG	Solution-phase synthesis non-GAG	Polymer (MPEG) supported synthesis
Name of associated companies and company websites	Organon (http://www.organon.com/) Sanofi-Aventis (http://www.sanofi-aventis.com/)	Organon (http://www.organon.com/) Sanofi-Aventis (http://www.sanofi-aventis.com/)	Organon (http://www.organon.com/)
Pros	Natural targets	Efficient More protecting groups available More reactive synthons	Efficient Opens up ways for automation
Cons	Lengthy Elaborate protecting-group strategy Synthons of lower reactivity	Non-natural derivatives	Difficult glycosylation procedures Reduced reactivity of building blocks
References	[2–5]	[4,5,12]	[15,25]

hexasaccharide was obtained after basic cleavage/deprotection in 37% yield.

Seeberger and co-workers [19] set out to develop a modular approach using disaccharide building blocks having the uronic acid at the reducing end, as is depicted in Fig. 4a–c. They developed a novel method for the construction of the α -D-glucosazide-D-glucuronic acid linkage, in which the glucuronic acid acceptors were locked in the 1C_4 -conformation [26]. Having constructed a collection of both glucosazido-iduronic acid (**37**, **38**, Fig. 4a) and glucosazido-glucuronic acid dimer building blocks (**39–43**, Fig. 4a), the modular journey towards larger heparin fragments was commenced. The tetrasaccharide stage was uneventfully reached providing a set of three highly diversified modules (**47–49**, Fig. 4b) for further elongation. In contrast to the success achieved in the above described syntheses of the Martín-Lomas laboratory [18,24,25], the Seeberger group [19] could not reach the hexasaccharide level using their envisaged [2 + 2 + 2] strategy. To get to the desired hexasaccharide fragment Seeberger and co-workers [19] rerouted their synthetic plan and continued with a [3 + 3] approach, relying on the stereoselective formation of the α -glucosazide-iduronic acid linkage (Fig. 4c). In the reported example, trisaccharide modules **50** and **51** were coupled to provide the highly functionalized protected hexasaccharide **52**, containing both glucuronic and iduronic acid residues. The unexpected failure of the [2 + 2 + 2] strategy can at the moment not be rationalized, but underscores the complex nature of GAG-synthesis [27].

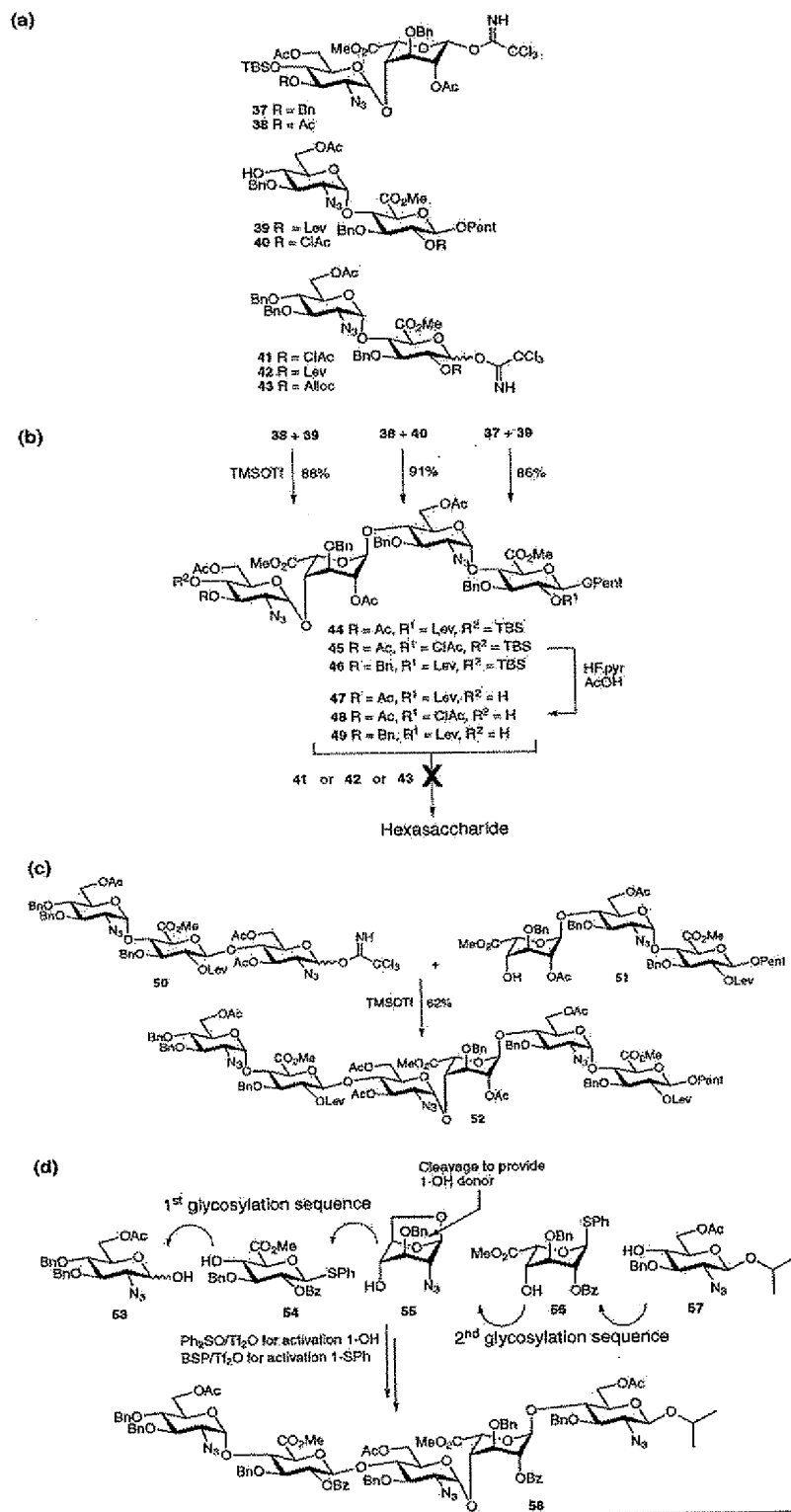
A conceptually different modular approach was developed in the van der Marel laboratory. To accommodate for the high degree of diversity in the H/HS structure an assembly strategy was developed that is based on the use of monomeric build-

ing blocks in a sequential glycosylation strategy (Fig. 4d) [28]. This will allow the future incorporation of a diverse set of building blocks in a straightforward manner and avoids the use of far-advanced and therefore precious dimer motives. Another difference with the approaches reported above is the use of 1-hydroxyl and 1-thiodonors in a field in which imidate chemistry is almost exclusively encountered. Key building blocks in the strategy are the 1-thio glucuronic and iduronic acids, **54** and **56**, respectively. These building blocks were synthesized in a highly efficient manner, exploiting a regio- and chemoselective oxidation of partially protected 1-thio glucose and idose synthons [29]. Condensation of the inactive thiodonors could be promoted by the use of recently developed powerful sulfonium activator systems [30,31]. A model pentasaccharide (**58**) was assembled in two consecutive condensation sequences from five monomeric building blocks (**53–57**) as is schematically depicted in Fig. 4d.

Conclusion

The strategies to access well-defined H/HS oligosaccharides each offer distinct (dis)advantages as summarized in Table 1. Comparing glycosaminoglycan H/HS synthesis to the assembly of non-GAG H/HS shows that the latter methodology offers great synthetic advantages: more temporary protecting groups are available, the building blocks used are more reactive, more robust (intermediate) structures can be obtained, and the deprotection/sulfation sequence at the end of the assembly is simplified. The evident setback of the non-GAG approach is that “non-natural” oligomers are generated. In the case of the anti-coagulant heparin pentasaccharide, this has proven to be no true disadvantage because it has been

Figure 3. Heparin and fibroblast growth factors (FGFs). (a) Structures of the FGF–FGFR–heparin complexes (reproduced with permission from [1]) [35]. The FGF is shown in green, the FGFR is depicted in orange. (b) Martín-Lomas’s modular strategy of FGF-binding heparin oligosaccharides in solution and on a soluble polymer support (PS, polystyrene solid support). (c) Synthetic heparin oligosaccharide with differentiated amino functionalities, depicted in red and blue.



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shown that highly biologically active pentasaccharides can be obtained. Interestingly, however, the use of the non-GAG saccharides to target other biological systems has only very scarcely been reported [32].

The modular approaches that have been recently reported in literature seem to be promising for the generation of a large variety of H/HS oligomers by the combination of different (dimer) modules. However, it has also become apparent that variations in the substitution pattern of the modules used can have a profound influence on the efficiency of the assembly and can mean the difference between failure and success.

Finally, it is appropriate to compare the polymer-supported assemblies to the corresponding solution-phase syntheses. Although the former technique should be a prelude to a true solid-phase approach and offers perspectives for future automation, it suffers from the inherent low reactivity of the building blocks used. In practice, the reported polymer-supported assemblies have not been shown to be a major improvement over the corresponding solution-phase approaches. The automated and combinatorial assembly of a large library of H/HS fragments is therefore not to be expected in the near future.

In summary, several formidable syntheses of H/HS-fragments have been recorded to date, culminating in the development of a fully synthetic antithrombotic drug. However, the assembly of highly diversified H/HS-oligosaccharides still presents a great challenge and the incorporation of the full array of variations in oligosaccharides of significant length remains a daunting task even for highly skilled specialists. It is expected that the development of innovative glycosylation methodologies and novel protecting groups will continu-

ously push the field forward and eventually can lead to a standard set of conditions for the modular assembly of a broad spectrum of H/HS-oligosaccharides.

Outstanding issues

- Differentiation of the amino functionalities. Although the differentiation of almost all hydroxyl functions in the heparin/heparan sulfate (H/HS) oligosaccharides can now be efficiently achieved, the differentiation of the amino groups still presents a major obstacle.
- Efficient solid-phase assembly of both non-glycosamino and glycosamino-glycans.
- Automated and combinatorial assembly techniques to access a large library of heparins and heparan sulfates.

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Figure 4. Modular approach towards heparin oligosaccharides. (a) Disaccharide modules used in the Seeberger laboratory. (b) Tetrasaccharide assembly and ensuing attempts towards the hexasaccharide via a [2 + 2 + 2] approach. (c) [3 + 3] strategy towards a highly functionalised hexasaccharide. (d) Monomeric building blocks in a sequential glycosylation sequence towards a heparin pentasaccharide (BSP, 1-benzenesulfinyl piperidine).

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